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Some Chemical and Physical Properties of Nisin, a Small-Protein Antibiotic Produced by *Lactococcus lactis*

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Nisin is a small gene-encoded antimicrobial protein produced by *Lactococcus lactis* that contains unusual dehydroalanine and dehydrobutyrine residues. The reactivity of these residues toward nucleophiles was explored by reacting nisin with a variety of mercaptans. The kinetics of reaction with 2-mercaptoethanesulfonate and thioglycolate indicated that the reaction pathway includes a binding step. Reaction of nisin at high pH resulted in the formation of multimeric products, apparently as a result of intramolecular and intermolecular reactions between nucleophilic groups and the dehydro residues. One of the nucleophiles had a pK_a of about 9.8. The unique vinyl protons of the dehydro residues that give readily identifiable proton nuclear magnetic resonances were used to observe the addition of nucleophiles to the dehydro moiety. After reaction with nucleophiles, nisin lost its antibiotic activity and no longer showed the dehydro resonances, indicating that the dehydro groups had been modified. The effect of pH on the solubility of nisin was determined; the solubility was quite high at low pH (57 mg/ml at pH 2) and was much lower at high pH (0.25 mg/ml at pH 8 to 12), as measured before significant pH-induced chemical modification had occurred. High-performance liquid chromatography on a C_{18} column was an effective technique for separating unmodified nisin from its reaction products. The cyanogen bromide cleavage products of nisin were about 90% less active toward inhibition of bacterial spore outgrowth than was native nisin. These results are consistent with earlier observations, which suggested that the dehydro residues of nisin have a role in the mechanism of antibiotic action, in which they act as electrophilic Michael acceptors toward nucleophiles in the cellular target.

Nisin is one of several ribosomally synthesized small protein antibiotics that contain dehydro residues (dehydroalanine [DHA] and dehydrobutryine [DHB] and thioether cross-linkages (lanthionine and β-methyllanthionine) that are introduced by posttranslational modifications of ordinary amino acids (serine, threonine, and cysteine). Structures are shown in Fig. 1. Although these antibiotics are produced by a disparate group of gram-positive bacteria (1, 5), their hydropathic and sequence homologies indicate that they have evolved from a common ancestor, which is supported by sequence homologies of their respective genes (3, 5). The conservation of the unusual residues over evolutionary time argues that they play a significant role in the biological function of these antibiotics. We have observed that nisin causes modification of the sulfhydryl groups in the envelopes of germinated spores (29), which is consistent with the dehydro residues acting as Michael acceptors toward sulfhydryl groups, as originally proposed by Gross and co-workers (12–14, 16).

Nisin is widely used as a food preservative in dairy products (18, 19). The fact that nisin is a posttranslational processing product of a gene-encoded peptide suggests that a variety of nisin analogs can be constructed by mutagenesis of the structural gene of the precursor peptide. For a variety of applications, these analogs might be superior to nisin itself. In order to make this a practical goal, it will be necessary to understand the posttranslational processing that converts serine, threonine, and cysteine into dehydro and lanthionine forms and the effects that these unusual amino acids have on the properties of the mature antibiotic peptide. Although nisin was discovered over 45 years ago (25), relatively little information about its fundamental chemical and physical properties is available. It is to be expected that nisin analogs will differ in properties such as solubility, chemical reactivity, and spectra. We are in the process of constructing nisin analogs. The purpose of this work is to obtain more information about the properties of nisin itself, in order to provide a reference for comparison when the analogs are available.

MATERIALS AND METHODS

Reagents. Highly purified nisin (>99%) was a gift of Applied Microbiology, Inc. (New York, N.Y.). Nisin solutions were stored in the dark, and all manipulations with nisin were performed in vessels that were wrapped to exclude light, to minimize light-induced free-radical reactions of the dehydro residues. Thioglycolate (TG) was obtained as 98% thioglycolic acid from Sigma Chemical Co., St. Louis, Mo.), and 2-mercaptoethanesulfonic acid (MES) was obtained as a 98% sodium salt from Aldrich Chemical Co., Inc., Milwaukee, Wis. D_2O (99.96 atom%) and deuterated trifluoroacetic acid (TFA) (99 atom%) were obtained from Aldrich Chemical Co.

HPLC chromatography and peptide sequence analysis. High-performance liquid chromatography (HPLC) was carried out on a Beckman System Gold HPLC system with a model 167 variable wavelength UV/VIS detector, a model 427 integrator, and a Beckman C_{18} Ultrasphere analytical column (inside diameter, 4.6 mm; length, 250 mm; packed with 5-μm beads with 8.0-nm pores). Gradient elutions with water-acetonitrile gradients (1.5 ml/min) containing 0.1% TFA were used. Typical gradients were 0 to 100% acetonitrile over 60 min (with nisin eluting at 27 min) and 50 to 100% acetonitrile over 30 min (with nisin eluting at 5.7 min). Peaks were monitored at 254 or 220 nm, as indicated, and quantitated by total area obtained by integration. The presence of peptide in the peaks was detected by spotting 10-μl volumes onto a piece of thin-layer chromatographic plate (0.2-mm

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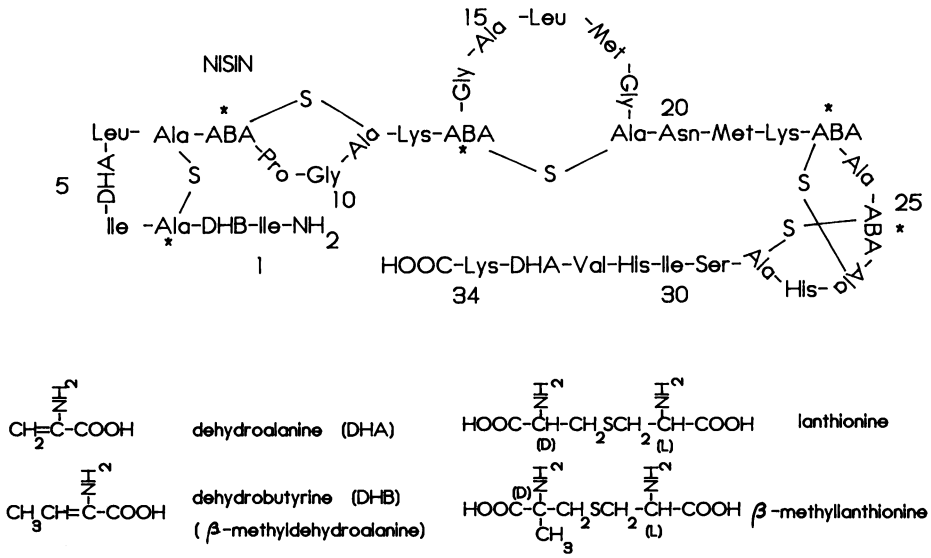

FIG. 1. Structure of nisin and unusual amino acids. Primary structure of nisin as determined by Gross (13). ABA, Aminobutyric acid; Ala-S-Ala, lanthionine; ABA-S-Ala, β-methyllanthionine. D-Stereo configuration (*) is shown for the α-carbon.

Silica Gel 60 on aluminum; EM Science), air dried for a few minutes, sprayed with ninhydrin (0.2% ninhydrin in *n*-butanol; reagent spray from Pierce Chemical Co., Rockford, Ill.), and heated at 95°C for about 4 min to develop the color. Peptide sequence analysis was carried out by using an Applied Biosystems model 477A automatic peptide sequencer with a model 120A analyzer.

Reaction of nisin at high pH and with mercaptans. Mercaptans used were MES and TG. Nisin was dissolved to a final concentration of 1.0 mg/ml in 50 mM NaP_i (pH 6.0). MES was dissolved at various concentrations up to 1.0 M, in the same phosphate buffer (that had been boiled to remove oxygen), and added to the nisin solution at 37°C to initiate the reaction, and the reactions were carried out in a water bath at that temperature. At selected reaction times, a 50-μl portion was removed, TFA was added to a final concentration of 0.3%, and 40 μl of the acidified mixture was injected onto the HPLC column, immediately washed with 0.1% TFA in water to quench the reaction, and then eluted with a gradient. The amount of reaction was determined from the area of the remaining nisin peak, and reaction rates were measured as disappearance of nisin. The reactions with TG were carried out in the same way, except that the buffer was 0.05 M sodium acetate (pH 3.8) at a temperature of 25°C. Reactions of nisin at high pH were done similarly, except that the only reaction components were nisin and water, buffered as indicated. After incubation at a selected pH, the reaction was stopped by acidification with TFA and the amount of reaction was quantitated as described above. Buffers used were NaP_i (pH 6.5 to 8.5 and pH 11 to 12), sodium carbonate (pH 9.1 to 10.1), or sodium borate (pH 9.6 to 10.6) at 50 mM. Observed rates were independent of buffer type. Each reaction rate was determined from the slope of a linear curve constructed from three to seven time points, with four being the usual number of time points and larger numbers of points being used for the faster rates.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Unreacted nisin was a stock solution (that had been stored at 4°C) containing 2 mg of nisin per ml in 0.1% TFA. Dilutions in deionized water were made prior to loading on the gel. Samples that had been reacted at high pH were neutralized with glacial acetic acid before diluting them with water. Samples that had been treated with MES were recovered from an HPLC gradient, lyophilized, and then dissolved in water. Electrophoresis was done by using the polyacrylamide gel system of Swank and Munkres, which is a sodium dodecyl sulfate-urea gel system that has been optimized for fractionation of small peptides (40). Gels were silver stained (#161-0443 kit; Bio-Rad Laboratories, Richmond, Calif.) by the protocol of the manufacturer.

Solubility of nisin. Saturated nisin solutions were obtained by dissolving an excess quantity of nisin in buffered water at 50°C; the resulting solution was vortexed for 1 min and centrifuged for 1 min in an Eppendorf microfuge at 12,000 rpm, and the supernatant was removed and allowed to cool to 25°C. It was then centrifuged again; appearance of precipitate at this step was evidence of saturation at 25°C. The amount of nisin in solution was determined by comparing the area of the nisin peak in an HPLC elution profile with that of a known quantity of nisin. Buffers used were 0.1% TFA (pH 2.1), 100 mM sodium citrate (pH 2.2 to 6.0), 50 mM NaP_i (pH 6.0 to 8.5 and pH 10.5 to 12.0), or 50 mM sodium carbonate (pH 8.5 to 10.5), unless indicated otherwise.

Cyanogen bromide cleavage of nisin. Cyanogen bromide cleavage of nisin was done as previously described (11, 15). A 33-mg portion of CNBr was added to 1-ml samples containing 3.3 mg of nisin per ml in 70% aqueous formic acid, incubated for various times at room temperature, and lyophilized. The residue was dissolved in 0.2 ml of water (pH between 1 and 2) and a portion was injected onto the HPLC column and eluted by a gradient to separate the cleaved fragments.

Assay of biological activity of nisin and its modified forms. Assay of biological activity of nisin and its modified forms was done as described by Morris et al. (27, 29). Heat-shocked spores of *Bacillus cereus* T (375 μg) were added to 1.0 ml of assay solution containing 1% tryptone (Difco Laboratories, Detroit, Mich.), 100 mM NaP_i buffer (pH 6.8), and various quantities of the material to be assayed. The samples were incubated in a rotating drum shaker (45 rpm) for 3 h at 37°C and examined by phase-contrast microscopy, which can distinguish between phase-bright ungerminated

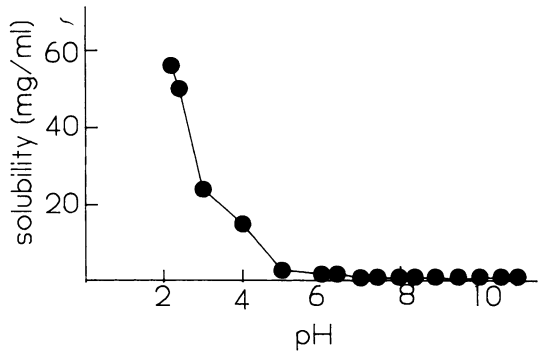

FIG. 2. Solubility of nisin in the pH range 2.2 to 11.5 at 25°C. The buffers used are described in the text.

spores, phase-dark germinated spores, phase-dark elongated spores, and vegetative cells. Uninhibited spores are vegetative within an hour, whereas in the presence of inhibitor, spores at various stages of development are seen. The amount of inhibitor was measured as the minimum quantity required to inhibit the spores in the elongated state (27, 29).

NMR spectroscopy. Nuclear magnetic resonance (NMR) spectra were obtained with a Bruker AF-400 (400-MHz) Fourier transform NMR spectrometer. Aqueous samples were lyophilized, suspended in D_2O, and lyophilized (three times) to exchange protons. The residue was then dissolved in D_2O or D_2O with 0.5% deuterated TFA. Depending on the concentration of the sample, 150 to 300 scans were collected. The HOD peak was suppressed by using the homonuclear presaturation (selective solvent suppression) technique with the PRESAT.AU program provided by IBM Instruments. The HOD peak was used as a reference.

RESULTS

Effect of pH on nisin solubility. Notable properties of nisin are that its solubility and stability increase dramatically as the pH is lowered. It is so stable at pH 2 that it can be autoclaved without inactivation (18), and we have stored refrigerated stocks of nisin in 0.5% TFA (pH 2.2) for months with no detectable chemical or biological changes. Because solubility often provides useful insight about the chemical nature of a substance, we looked at the solubility of nisin from pH 2 to 12 (Fig. 2). The effect of pH on solubility was indeed dramatic. The solubility dropped sharply and continuously from 57 mg/ml, at pH 2, to about 1.5 mg/ml, at pH 6; it dropped again to 0.25 mg/ml, at pH 8.5, whereupon it leveled off. It is interesting that the pH at which solubility leveled off (about pH 8) coincides with the pH at which nisin began to undergo pH-induced modifications (see below), although the significance of this is not clear. Solubility measurements were obtained before significant pH-induced chemical modification had occurred.

Buffer concentration affects nisin solubility, and we observed that the solubility of nisin is inversely and linearly proportional to phosphate buffer concentration, being 42% less (at pH 7.0) for 0.2 M NaP_i than for 0.05 M NaP_i (data not shown). This buffer concentration effect was taken into account in determining the pH versus solubility profile, in that the pH ranges for successive buffers were overlapped and the solubilities in the overlapping regions were established to be the same.

Effect of pH on nisin stability. It has long been known that nisin is unstable and becomes inactivated at high pH (18). The mechanism of inactivation is unknown but could be a consequence of denaturation, chemical modification, or a combination of both. The dehydro residues are potentially susceptible to modification by nucleophiles that are present at high pH, such as hydroxide ions, deprotonated amines, and deprotonated hydroxyl groups. Reactions with these nucleophiles could be intramolecular or intermolecular, the latter causing cross-linking. Since there are three dehydro residues per molecule, large multimolecular aggregates could form by intermolecular reactions (17).

Nisin was incubated under various conditions of pH, and the extent of modification was evaluated by HPLC and electrophoresis of the reaction products. Figure 3 shows the rate at which the nisin peak disappeared. The biological activity of the material in the nisin peak remaining during the reaction was determined by its ability to inhibit bacterial spore outgrowth; the specific activity remained constant, indicating that the nisin peak represented material that had not undergone irreversible chemical or physical changes. The pH-dependent reaction rate was quite low below pH 8, whereas it rose rapidly above pH 8. Figure 3 shows at least two components of the reaction, one that rises rapidly between pH 8 and 10 and another that rises linearly with hydroxide ion concentration. Both rates extrapolate through zero, indicating that the reaction is dependent on hydroxide ion. If one assumes that the initial rise is due to a single titratable group that becomes sufficiently nucleophilic when deprotonated to react with a dehydro residue, that group has a pK_a of about 9.8. Any of several amino groups in nisin are good candidates. At the higher pH values, the fact that the rate does not level off, even at a pH value of 12, suggests that the hydroxyl group itself is the predominant nucleophile at high pH, although involvement of other nucleophiles is not precluded.

We conclude that inactivation of nisin at high pH is not a consequence of simple denaturation, because there is not the sharp pH transition that is typical of a denaturation process. Conversely, a nisin molecule at elevated pH undergoes modification with kinetics that are consistent with a chemical process, and it appears that unmodified nisin rapidly attains its normal configuration when the pH is changed from high pH to low pH, as evidenced by the fact that its HPLC profile and specific activity are unaffected by this treatment.

The physical and chemical nature of the material that had undergone reaction at pH 11.0 was examined further. Its solubility characteristics were dramatically changed. The solution became turbid during the reaction, indicating the formation of an insoluble reaction product. A portion of the soluble fraction was fractionated by HPLC (data not shown). There are many reaction products formed under these conditions, some which elute before nisin (more polar than nisin), and some which elute after nisin (less polar than nisin). A major portion elutes in a multicomponent peak before nisin. Figure 3 suggests that much of the reaction at pH 11 involves reaction with hydroxyl groups, which should increase polarity. This is consistent with the observed result. The more hydrophobic products may reflect reaction with other nucleophilic groups, in either inter- or intramolecular reactions. The nature of the reaction products obtained after treatment at high pH was examined further by polyacrylamide gel electrophoresis (Fig. 4). The gel reveals several products with sizes that correspond to multimers of nisin; the intensity of the dimer-size band was about equal to that of the monomer-size band, with fainter bands corresponding to the sizes of trimer, tetramer, and pentamer. There is also

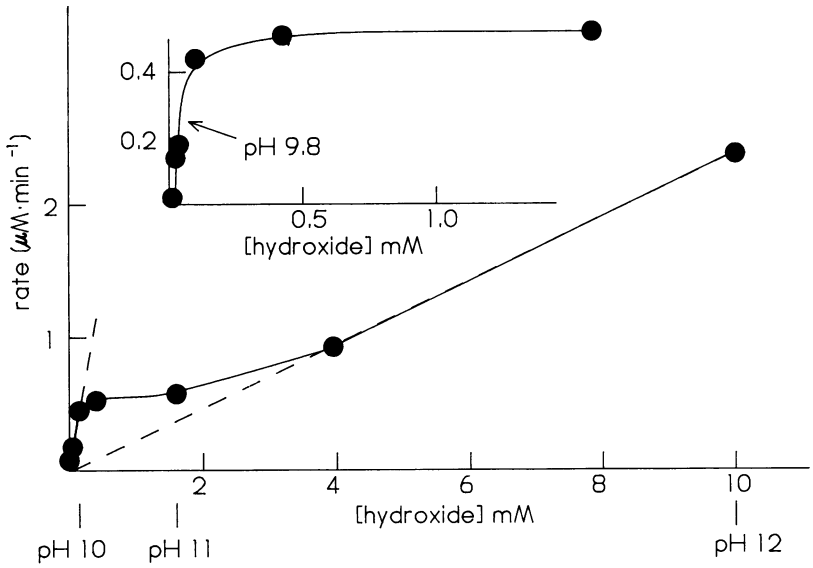

FIG. 3. Nisin reaction at high pH. The rate of disappearance of nisin at different hydroxide ion concentrations and on an expanded linear scale (inset) are shown. Experimental reaction times varied from more than 12 h at pH 6.5, to 10 h at pH 9.0, to 50 min at pH 12.0.

a faint band that corresponds to the dimer position in the unreacted nisin lane. The HPLC profile of these same products showed that almost all of the nisin had undergone reaction, so the monomer-size band in Fig. 4, lane 2, cannot represent nisin that had not yet reacted but represents nisin that had reacted in a way that did not significantly change its electrophoretic mobility on this sodium dodecyl sulfate-urea gel. On the other hand, the larger products seem likely to be nisin multimers. Monomers could arise as reaction products from hydroxyl addition to dehydro groups or from intramolecular additions involving nucleophilic groups within the peptide. Multimers could arise from a variety of intermolecular reactions, such as nucleophilic R groups from one nisin molecule adding to dehydro groups of other nisin molecules. Free-radical reactions involving dehydro residues to give either mono- or multimeric forms are also possible, but the reaction conditions employed here, which included excluding light during manipulations, would not favor them. Other reactions are also possible.

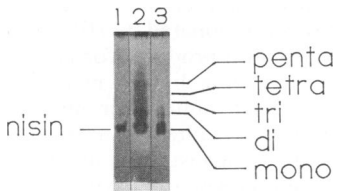

FIG. 4. Electrophoresis of reacted nisin. Lane 1, Unreacted nisin; lane 2, nisin after reaction for 12 h at 37°C in 50 mM NaP_i at pH 11; lane 3, nisin after reaction with MES, as described in the legend to Fig. 5 (the HPLC profile of this sample is shown in Fig. 5B, and the NMR spectrum is shown in Fig. 6D). Nisin has an M_W of 3,354. On the basis of size estimates from protein standards, the expected positions of bands corresponding to the various mono- and multimeric forms of nisin are shown. A faint band corresponding to the size of nisin dimer appears in both lanes 1 and 3. An intense dimer-size band appears in lane 2, along with fainter bands corresponding to trimer, tetramer, and pentamer.

Reaction of nisin with mercaptans. The dehydro residues of nisin, in which the double bond is conjugated with the peptide carbonyl (Fig. 1) are typical Michael acceptors, which should react readily with mercaptans and other good nucleophiles (4, 10, 13, 15, 35, 41). Although the reaction of nisin with mercaptans has been demonstrated, no kinetic information is available. The reaction kinetics were explored by using MES, TG, and other mercaptans. Incorporation of the charged MES by reaction of the sulfhydryl group of MES with a dehydro residue would cause the adduct to be more polar and to elute earlier in the HPLC gradient than unmodified nisin (Fig. 5). It seems unlikely that the poorly nucleophilic sulfonic acid group would add to a dehydro group. The reactions of various concentrations of MES with a constant concentration of nisin are also shown in Fig. 5. The velocity of reaction of MES with nisin increases up to a concentration of about 40 mM MES, after which the reaction rate levels off, suggesting that a binding site has become saturated. NMR spectroscopy of the product of MES with nisin shows that the dehydro residues of nisin have undergone reaction, and the proton resonances of all three dehydro residues are lost (see below; Fig. 6). TG underwent reaction with nisin in a similar way (Fig. 5). In the reaction with TG, the pH and temperature were lowered with respect to the conditions used with MES in an attempt to suppress involvement of the carboxyl group of TG, which is more strongly nucleophilic than the sulfonic group of MES. The results in Fig. 5 show that the rate profiles with TG and MES are similar, with both rates leveling off at about 40 mM mercaptan. The sizes of the products formed after reacting nisin with MES were examined by using polyacrylamide gel electrophoresis (Fig. 4). In contrast to the results at high pH (Fig. 4), the reaction products of nisin with MES were of a similar size as those of unreacted nisin, although the products did not electrophorese as a single discrete band. Inasmuch as there was heterogeneity among the reaction products, as shown by HPLC, heterogeneity as shown by electrophoresis is not surprising and is consistent with there being various numbers of dehydro residues that have undergone reaction with

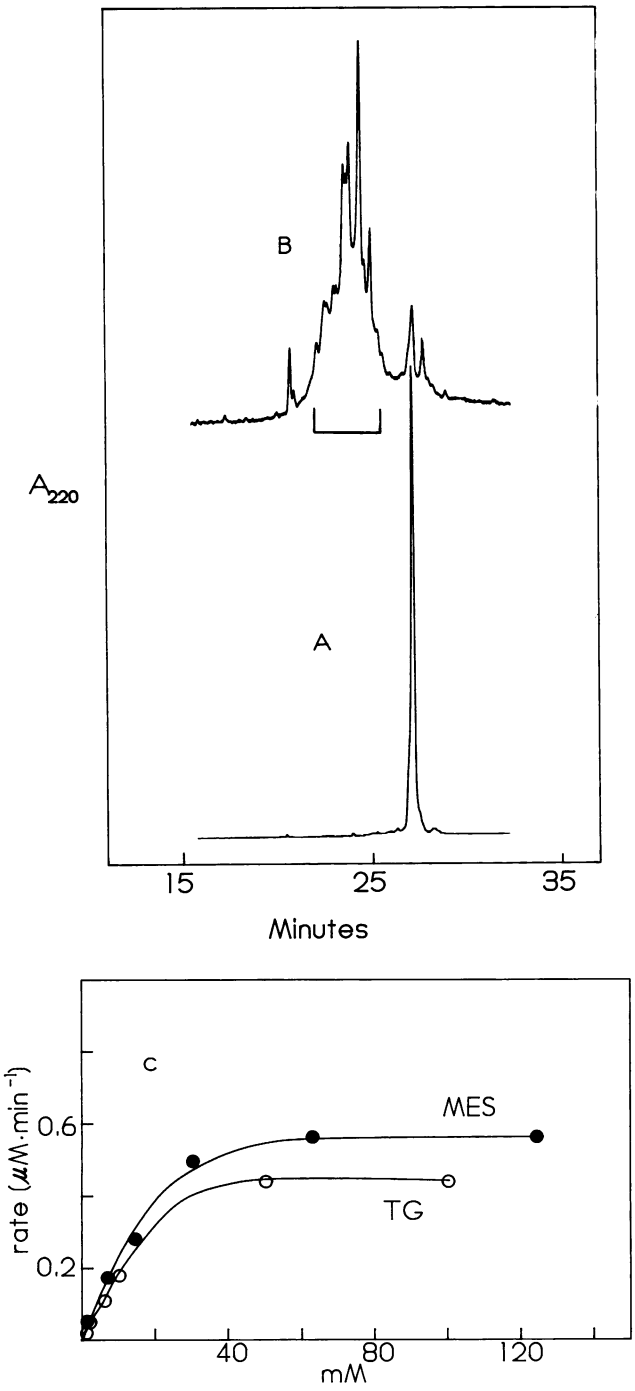

FIG. 5. Reaction with mercaptans. HPLC profiles (220-nm wavelength) of the products of reaction of nisin with 100 mM MES after different reaction times at pH 6.0, in 50 mM sodium [2-(*N*-morpholino)ethanesulfonic acid] buffer at 37°C are shown. (A), reaction time of 0 min (i.e., unreacted nisin); (B) reaction time of 38 h. When nisin was incubated in the same buffer for 24 h in the absence of MES, the nisin peak did not change (data not shown). The gradient was 0 to 100% acetonitrile in 0.1% TFA for 60 min, as described in the text. The bracketed region in profile B was pooled and analyzed by NMR spectroscopy (Fig. 6B), electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel (Fig. 4), and tested for the presence of peptide with ninhydrin. Both the NMR spectrum and the ninhydrin reaction showed the presence of peptide. (C) Reaction rates of MES with nisin in 50 mM NaP_i at pH 6.0 and 37°C and the reaction rates of TG with nisin in 50 mM sodium acetate at pH 3.8 and 25°C.

the mercaptan. There is also a small amount of dimer-size material among the reaction products of MES with nisin. This could merely be the same material as the faint band that was present in unreacted nisin, or it could reflect a pH-induced reaction product (the pH-induced rate is not zero at pH 6), or it could be an MES-induced dimerization. Since it is such a minor reaction product, an attempt to distinguish among these possibilities was deferred.

NMR spectroscopy. Nisin is particularly suitable for proton NMR studies, because the vinyl protons in the dehydro residues give resonance signals that are easily distinguished and well separated from those of other protons in the peptide. Recent two-dimensional NMR studies have shown that the nisin molecule adopts a well defined three-dimensional structure (37). Other workers have characterized the vinyl protons in DHA and DHB, both as small molecules (2; C. L. Fisk, Ph.D. thesis, Georgetown University, Washington, D.C., 1975) and in peptides (2, 7, 22–24, 26, 31, 32, 36). The peak assignments of DHB and DHA are determined according to several criteria. The two vinyl protons of monomolecular DHA have been observed to give two peaks (20; Fisk, Ph.D. thesis), and the single vinyl proton of monomolecular DHB has been observed to give a quadruplet (7; Fisk, Ph.D. thesis), which is consistent with expectations on the basis of their structures. The differences in the chemical shifts of the vinyl protons in DHA_{33} and DHA_5 are due to the differences in their respective peptide environments, and the significant spectral differences observed suggest that these environmental differences must be fairly large. The DHA_{33} peak has been distinguished from the DHA_5 peak by CNBr cleavage of nisin into fragments containing amino acids 1 through 21 and 22 through 34 and by determining which resonances are present in each (Fisk, Ph.D. thesis). Our results show that there are changes in the spectrum when the pH is changed from neutral to acidic. Under acidic conditions, there is a pronounced upfield shift of the DHA_{33} resonances, which may be due to an ionization change in the adjacent C-terminal lysine residue, which could in turn induce conformational or other changes. Moreover, the areas of the DHA_{33} peaks decrease under acidic conditions, indicating that they have shifted to a different environment and perhaps reflecting a conformational change (9, 32). As pH was changed, the change in the DHA_5 peaks was minimal. This is not surprising for two reasons: DHA_5 is flanked on both sides by nonionizable groups, and it is also in a conformationally stabilized ring structure that is maintained by a thio-ether cross-link. That the chemical nature of DHA_5 is different from that of DHA_{33} has been established by the observation that DHA_{33} is more susceptible to degradative acid hydrolysis than DHA_5 (8). Another significant change in the spectrum that occurs at low pH is that the DHB_2 quadruplet is considerably diminished in size; this spectral change is reversible, and the original spectrum returns (data not shown) upon neutralization. Since nisin is unaffected by extended exposure to low pH, these pH-induced spectral differences must reflect reversible conformational and charge effects and not degradation or other irreversible changes.

Sporostatic activity of CNBr-generated nisin fragments. Gross and Morell reported that CNBr cleaves nisin into two fragments (fragment 1 through 21, which contains DHB_2 and DHA_5, and fragment 22 through 34, which contains DHA_{33}) and that both fragments retain biological activity against bacterial cells (17). Although both fragments were active, no information about changes in specific activity was reported. Our activity assay can quantitatively measure the activity

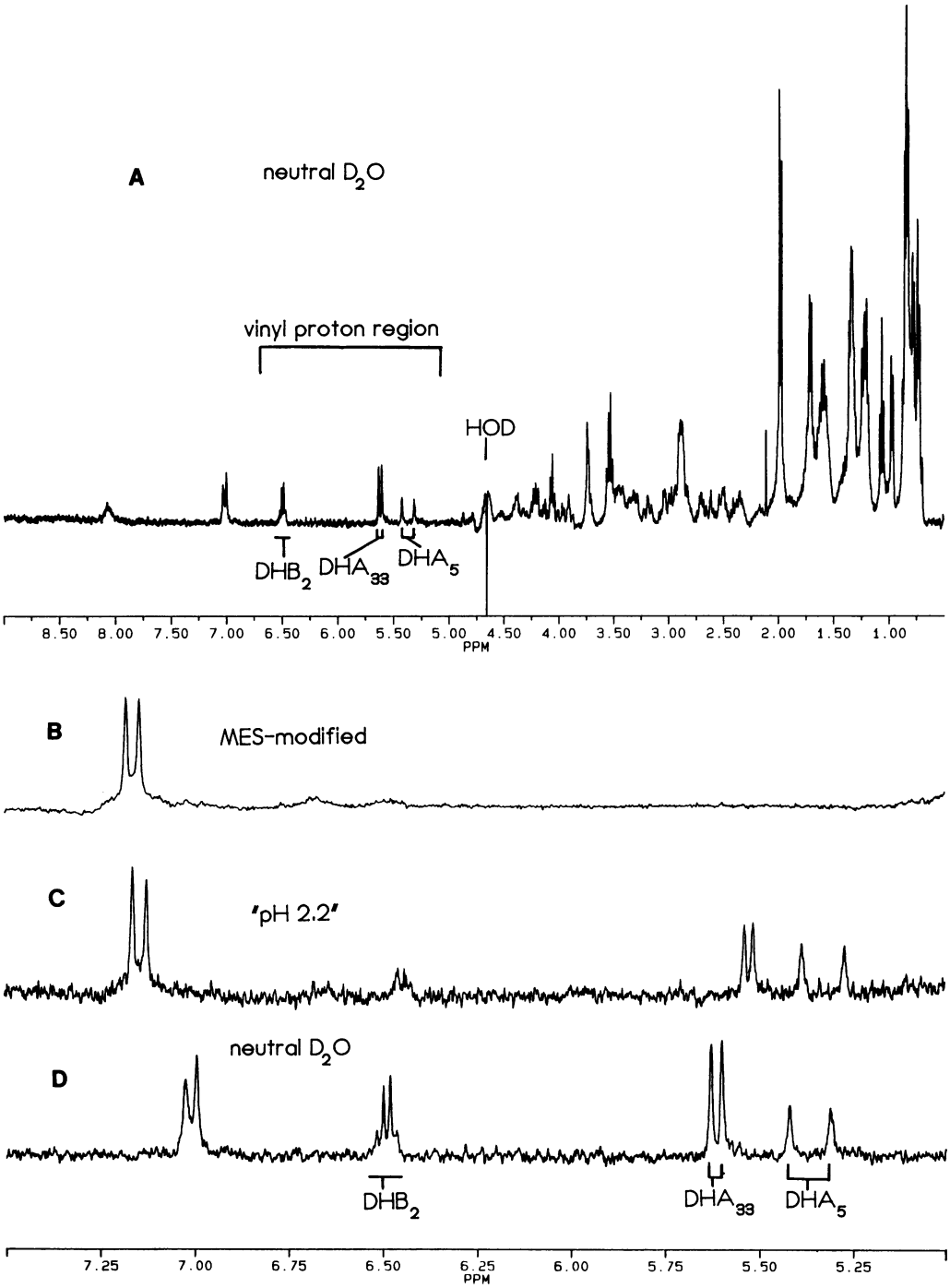

FIG. 6. Proton NMR spectra (400 MHz). (A) Complete proton NMR spectrum of nisin in neutral D_2O. Profiles B through D show expanded-scale partial spectra in the region where the unique resonances of the vinyl protons of DHA and DHB appear. (D) Nisin in neutral D_2O; (C) nisin in D_2O containing 0.5% deuterated TFA; (B) spectrum (solvent as in profile C) of nisin after reaction with MES (Fig. 5).

against outgrowing bacterial spores. Nisin was cleaved with CNBr (46-h reaction) and the two major fragments (equimolar, as judged by peak area) were purified by HPLC. Both fragments were able to inhibit spore outgrowth when assayed in our system; however, the specific activities of both fragments were diminished at least 10-fold in comparison with native nisin. We do not have enough information to establish the reason for activity reduction, but it appears that activity is not absolutely dependent on the simultaneous presence of all three dehydro residues in the same peptide molecule. On the other hand, extensive reaction of nisin with mercaptans or at elevated pH (Fig. 3 through 6) modifies the dehydro residues and inactivates nisin, suggesting that dehydro residues are important for activity.

DISCUSSION

It is an understatement to say that the properties of nisin are unusual for a ribosomally synthesized peptide. Post-translational processing converts 13 of 34 residues into unusual forms (electrophilic dehydro residues and thio-ether cross-linkages) that have chemical characteristics that are not possessed by any of the normal gene-encoded amino acids. We therefore cannot draw upon experience to assess whether the properties and behavior of these residues are intrinsic or whether they are significantly modified by their chemical environment within the peptide. As is usually the case with nature versus nurture, it appears to be a combination of both. It is clear that the differential peptide environments around DHA_5 and DHA_{33} are responsible for their differences in the proton NMR spectrum. It is much less clear to what extent the peptide environments around the dehydro residues influence their chemical reactivities. It has recently been shown that an intact DHA_5 is required for the N-terminal (residues 1 through 32) portion of nisin to possess biological activity (8). Our working hypothesis, which is supported by earlier observations (16) as well as this work, is that the dehydro residues play an important role in the mechanism of nisin antibiotic action by reacting with one or more nucleophiles in a sensitive cellular target. We have shown that nisin inactivates sulfhydryl residues in germinated bacterial spores (29) in a manner that is similar to those of some other sulfhydryl-directed agents (6, 27–29). It seems likely that the chemical reactivity of nisin consists of two components: reactivity toward a specific cellular target nucleophile and reactivity toward nucleophiles in general.

With this in mind, we chose simple mercaptans (MES and TG) in order to look at nisin reactivity with some general nucleophiles. The reaction kinetics with nisin were first-order with respect to mercaptan, until a maximum rate was attained with 40 mM mercaptan. This suggests that the mercaptan must bind before reacting and that the binding site can become saturated. It is possible that this same site is responsible for binding to the cellular target, although this is speculative. In contrast, the hydroxyl ion does not show saturation, indicating that its small size may allow it to approach the dehydro residues without having to bind to a particular site. It is also of interest that the products of reaction of nisin with mercaptans were monomeric, whereas the products at high pH consisted largely of dimers and other multimers. This can be rationalized by assuming that the mercaptan simply added across the double bond of the dehydro residues, whereas high pH activated potential nucleophiles such as amino groups (13) by deprotonation. If so, after extensive modification at high pH, the presence of monomer-sized products must reflect either adducts with hydroxyl ion or adducts involving intrapeptide nucleophiles; whereas dimers and multimers reflect involvement of interpeptide nucleophiles. Evidence is presented that one of the nucleophiles has a pK_a of about 9.8, but its identity has not yet been established.

There is a growing body of evidence that α,β-unsaturated amino acids such as DHA and DHB are important in many biological systems and that they occur in a wide variety of natural products that have antibiotic properties (19, 30, 33–35), although little is known about the mechanistic roles that they play. Whereas α,β-unsaturated amino acids are introduced into biological molecules by a variety of biosynthetic pathways (21, 30, 35, 38, 39), those in nisin are determined by the sequence of the nisin structural gene, which permits the use of genetic approaches for the study of their properties and functions. This work is in progress.

ACKNOWLEDGMENTS

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